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Effect of *Lactobacillus salivarius* H strain isolated from Chinese dry-cured ham on the color stability of fresh pork

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Abstract

The aim of this research is to investigate the effect of lactic acid bacteria (LAB) on color stability of fresh pork. Of the seven LAB strains isolated from the Chinese dry-cured hams, H strain was identified as *Lactobacillus salivarius* (CCTCC M2010374) by serial biochemical tests and 16S rRNA analysis. The fresh pork samples were immersed in the H strain and *Lactobacillus fermentum* extracts for 1 min after ultrasonic cells disruption and samples immersed in sterile 0.9% (*m/V*) saline was served as a control group, then samples were vacuum packaged and stored at 4 °C. Nitrate oxide synthase (NOS) and metmyoglobin (MetMb) reductase were found in the cells extracts of H strain. After 6 days of storage, H strain group was found to be associated with the highest CIE a* values ($P < 0.05$) and the lowest MetMb (41%). *L. salivarius* H strain has thus shown a beneficial effect on the color stability of fresh pork. Further research is necessary to elucidate its mechanism.

Keywords: Pork; Color stability; *Lactobacillus salivarius*; Metmyoglobin

1. Introduction

Visual appearance is of vital importance for the quality of most food products, with color of (as) utmost importance as it affects consumers when they evaluate freshness and quality of meat and meat products [1–6]. The meat industry has employed different ways of protecting the red color in meat and the most commonly used approach is the modified atmosphere packaging (MAP) with different gas concentrations (CO_2, O_2 and N_2) [7].

Carbon monoxide (CO) is also reported to have a significant effect on color stability of fresh meat [3,8,9]. The use of CO in packaging has become increasingly relevant for the US meat industry since its approval by FDA [10–12] at a level of 0.4% in red meat MAP systems [3]. However, it is one of the most dangerous gases among air pollutants and in daily life. It is extremely toxic and dangerous because it is colorless and odorless. The fact that CO can deceive consumers creates an unnecessary risk of food poisoning by enabling meat and ground beef to remain fresh-looking beyond the point at which typical color changes that indicate aging or bacterial spoilage. Thus, the use of CO remains a question and it seems that an alternative method is required to replace it.

According to Morita et al., NO, which is responsible for color formation in nitrite-free cured meat, was formed from *L*-arginine due to nitric oxide synthase (NOS) in either *Staphylococci* or *Lactobacilli* [13,14]. Arihara et al. found that *Lactobacillus fermentum* JCM1173 can transform Mb (Fe^{3+}) (Mb, Myoglobin) into cured meat pigment NO-Mb (Fe^{2+}) [15]. Morita studied 10 *L. fermentum* strains and found that all of them can transform Mb (Fe^{3+}) into bright red NO–Mb (Fe^{2+}) in the MRS (Man–Rogosa–Sharpe) culture medium, and *L. fermentum* IFO3956 had an outstanding transforming ability, which can utilize NOS to form NO from *L*-arginine [13]. Another study on production of cured meat color in nitrite-free sausages by *L. fermentum* showed that nitrosylmyoglobin could

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be generated when *L. fermentum* AS1.1880 was inoculated into the meat batter, and the formation of a characteristic pink color with an intensity comparable to that in nitrite-cured sausage can be achieved using 10^8 CFU/g of the culture [2].

To date, however, no research has evaluated the effects of lactic acid bacteria (LAB), the generally regarded as safe (GRAS) strains, on the enhancement of fresh pork color.

Therefore the objectives of this study are (1) to isolate and characterize a potential strain from the Chinese dry-cured hams, (2) to investigate the effects of the strains on the color stability of fresh pork.

2. Materials and methods

2.1. Materials

The commercially available Jinhua ham, a Chinese dry-cured ham, and fresh pork were purchased from a local market in Guangzhou.

Lactic acid bacteria (LAB): *L. fermentum* JCM1173 and *Lactobacillus salivarius* LH1F were isolated by the Applied Microbiology Laboratory of South China Agricultural University for probiotic purpose. *Escherichia coli* DH5a was used as the host strain.

Indicator pathogens were:

E. coli (ATCC 11775); *Salmonella typhimurium* (ATCC 1769NR); *Staphylococcus aureaus* (ATCC 13563).

Media: MRS or MRS + $CaCO_3$ medium for acid prodution testing; amino acid decarboxylase medium; arginine dehydrolase medium; H_2O_2 generation detection medium; PY basal medium; PYG medium.

2.2. Experimental methods

2.2.1. Isolation and screening of lactic acid bacteria (LAB) strains

The fermented meat (Jinhua ham) was cut into pieces of 25 g followed by further cutting using a pair of scissors to increase the surface area. 225 mL saline was added and the suspension was shaken for thorough mixing. Serial dilution was done; 0.1 mL of dilution was spread on MRS + $CaCO_3$ agar plates, which were incubated for 48 h at 37 °C under anaerobic condition.

The colonies formed on the agar plates were picked up as candidates of LAB strains. Seven LAB strains were selected from Jinhua hams based on the demonstrated differences of H_2O_2 and H_2S production ability, amino acid decarboxylase production activity, *L*-arg ammonia production ability and antibacterial activity against common food-borne pathogens such as *E. coli*, *S. typhimurium* and *S. aureaus*. The antibacterial activity of LAB candidate strains was tested according to Pavente et al. [16].

2.2.2. Identification of LAB

Analytical Profile Index (API) identification system, including physical, biochemical, and molecular biological characteristics tests, was used for the preliminary identification of the isolated LAB.

16S rRNA phylogenic analysis was also conducted for LAB strain identification. Total chromosomal DNA was purified from the LAB strains' cells growing in proper conditions according to the method used in previous reports [17], and then used as a template for polymerase chain reaction (PCR) amplification (Takara PCR thermal cycler TP600, Takara Bio Inc., Japan).

The 16S rRNA gene was amplified by PCR using universal bacterial 16S rRNA forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal reverse primer 1541R (5′-AAGGAGGTGATCCAGCCGCA-3′) [18]. PCR amplications program comprised 30 cycles, with denaturation at 94 °C for 45 s, primer annealing at 65 °C for 30 s and extension at 72 °C for 1.5 min. The amplified product was cloned in pMD18-T vector (TAKARA) and *E. coli* DH5a was used as the host strain. The recombinant pMD18-T-16S vector containing 16S rRNA amplified from H strain was sent to Invitrogen Biotechnology (Shanghai) Co., Ltd. for sequencing. The BLAST program (NCBI, USA) was used for sequence comparison. Phylogenetic and molecular evolutionary analyses were conducted by clustalX v1.81. Distances and clustering were calculated using the distance-based method. Bootstrap analysis was used to evaluate the tree topology of the distance-based data by performing 100 resamplings.

2.2.3. Preparation of LAB cells suspension for enzymes extraction

L. salivarius H strain and *L. fermentum* JCM1173 cells were cultured for 18 h during the early stationary growth phase and harvested by centrifugation at 3000 r/min (1000 × *g*) for 20 min at 4 °C (Centrifuge 5804R, Eppendorf, Germany). Cell pellets were washed twice with saline (pH 7.0). 5 g (wet weight) cells were re-suspended in 20 mL saline (pH 7.0).

In order to extract the intracellular enzymes from LAB, the cells suspensions in saline were subjected to ultrasonic cell disruption twice (Sonics & Materials. INC, VCX 750, and USA) without output power 750 W, amplitude 30%, pulse, operation time 10 min, temperature 40 °C.

2.2.4. Determination of nitric oxide synthase (NOS, EC 1.14.13.39) activity

NOS activity was measured in the bacterial extract from inactivated H strain according to the method described by Chen and Rosazza [19]. The consumption of nicotinamide adenine dinucleotide phosphate (NADPH) was determined by the decrease in absorbance at $\lambda = 40$ nm as NADPH is oxidized during the conversion of *L*-arginine to *L*-citrulline. NOS activity was expressed as the difference between NADPH consumption with the substrate of *L*-arginine and NADPH consumption without *L*-arginine.

The NOS assay reaction system (total volume 1 mL) was composed of phosphate buffer (50 mmol/L; pH 7.0), $CaCl_2$ (1.0 mmol/L), flavin adenine dinucleotide, disodium salt (FAD) (10 mmol/L), flavin mononucleotide, monosodium salt dihydrate (FMN) (10 mmol/L), NADPH, tetrasodium salt (1.0 mmol/L) and 0.5 mL LAB cells suspension. The reaction was started by adding *L*-arginine (1.0 mmol/L).

Assays were performed at 30 °C for 30 min with stirring. After that the absorbance at 340 nm wavelength was measured within 15 min. The group without *L*-arginine was set as the blank. The content of NADPH was obtained from a standard curve.

2.2.5. Metmyglobin (MetMb) reductase activity

The metmyoglobin reductase activity was measured according to the assay described by Mikkelsen et al. [20] and Bekhit et al. [21]. The assay reaction system (total volume 1 mL) was composed of 0.1 mL 5.0 mmol/L Na_2EDTA, 0.1 mL 50.0 mmol/L sodium citrate buffer solution (pH 5.65), 0.1 mL 3.0 mmol/L potassium ferrocyanide, 0.2 mL 0.75 mmol/L metmyoglobin reductase substrate, 0.2 mL H_2O, 0.2 mL LAB cells suspension, and 122.0.1 mL 1.0 mmol/L NADH (pH 7.0). The mixture (pH 6.4) was at 30 °C.

The reaction was initiated by adding NADH. The absorbance at 580 nm wavelength was measured every 15 s altogether 2 min until the difference of optical density between MbO_2 and MetMb reached the maximum. The molar extinction coefficient was 1.2×10^4.

2.2.6. Sample preparation for meat color stability

Meat from pectoralis major and minor muscles of pork carcasses 48 h postmortem were purchased from a local market in Guangzhou. All exterior fat and connective tissue was trimmed off in sterile conditions and cut into 25 g pieces.

The pork samples were divided into 3 groups with 9 pieces in each group. (1) Control group treated with sterile 0.9% (*m/V*) saline; (2) H strain group; and (3) *L. fermentum* JCM1173 group.

The meat pieces were immersed in LAB extracts (described above in Section 2.2.3) and saline for 1 min then removed and left to drip for 1 min followed by vacuum packaging and storage at 4 °C for 6 days.

2.2.7. Sensory evaluation

An evaluation team of 10 people who went through a specialized training recorded the changes in color, appearance and odor and evaluated all the treatment samples according to the marking standards [22].

2.2.8. Surface color measurements

Measurement of meat surface reflectance, expressed as CIE L^* and a^* values, was performed on meat slices with a thickness of approximately 3 cm during the 6 days storage period at 4 °C using Chroma meter CR-410 (Konica Minolta Sensing Inc., Japan). The three samples were measured in triplicates. The measurements were taken around the center of the meat pieces and average values of the CIE L^* and a^*values were recorded, according to Tang & Shelef [23].

2.2.9. Metmyoglobin (MetMb)

The meat samples (5 g) were homogenized in 25 mL ice-cold 40 mmol/L phosphate buffer (pH 6.8) for 1 min using an Ultra-Turrax T25 tissue macerator (13,500 r/min). The homogenate was allowed to stand for 1 h at 4 °C and centrifuged at 4500 g for 30 min at 4 °C. The supernatant was filtered through a filter paper and the absorbance was measured at 572, 562, 545 nm and 525 nm using a Shimadzu UV-2101PC spectrophotometer (Shimadzu Inc., Columbia, MD) connected to a computer. The percentage of MetMb was determined according to Krzywicki [24] using the formula below:

$$\%\text{MetMb} = \left\{-2.51\left(\frac{A_{572}}{A_{525}}\right) + 0.777\left(\frac{A_{562}}{A_{525}}\right) + 0.8\left(\frac{A_{545}}{A_{525}}\right) + 1.098\right\} \times 100$$

2.2.10. Statistical analysis

It was a randomized experiment with triplicates. The data obtained from analyses were subjected to variance analysis in order to determine the effect of *L. salivarius* H strain and storage time on each variable. The analysis was performed by ANOVA one-way analysis, using 'SPSS-12.0' software packages as per the standard methods of Snedecor and Cochran [25]. To identify the different groups, the Duncan's *post hoc* test was applied.

3. Results and discussion

3.1. Isolation and primary screening of LAB strains

Seven different Gram positive bacteria strains were selected based on the size of calcium dissolving zone on MRS agar plates, morphological characteristics, colonial type and Gram's staining. Among them X11 was *Cocci* while the other 6 strains were *Bacilli*.

3.2. Secondary screening of LAB strains

Results of secondary screening tests are shown in Tables 1 and 2.

In Table 1, there was no amino acid decarboxylase, and neither H_2S nor H_2O_2 were produced during growing in H strain. Moreover, it fermented glucose without air consumption (aerogenesis). The absence of H_2S and H_2O_2 showed that H strain can prevent risks of green discoloration of meat and bacterial contamination.

Compared to other strains, H strain cultural broth showed an outstanding antibacterial activity against common meat contaminating and spoiling bacteria. The broth of H strain can inhibit the growth of *Salmonella typhimurium*, *S. aureus* and *E. coli in vitro* with a inhibition zone diameter of 1.85 ± 0.0071, 2.10 ± 0.0047 and 2.10 ± 0.0067 cm respectively (Table 2).

Table 3 indicates the presence of nitric oxide synthase and metmyoglobin reductase in H strain extracts. Although some researchers have found no or negative correlation between MetMb reducing activity and color stability [26,27], others concluded that MetMb reductase was actively involved in the reduction of MetMb in tuna meat during cold storage while more studies suggested that the MetMb reductase activity was the controlling factor in retardment of the accumulation of MetMb

Table 1
Biochemical reaction test results of candidate strains.

Candidate strains	Amino acid decarboxylase	Glucose aerogenesis	H_2O_2 production	H_2S production	L-Arg ammonia production
X1	+	+	−	−	−
X8	+	−	−	+	+
X9	+	+	+	−	−
X11	+	−	+	−	−
X15	+	+	+	+	+
L. salivarius LH1F	−	−	−	−	+
H	−	−	−	−	−

+: positive reaction; −: negative reaction.

Table 2
Antibacterial activity of LAB candidate strains.

Candidate strains	Diameter of inhibition zone (cm)		
	Salmonella typhimurium (ATCC 1769NR)	*Staphylococcus aureus* (ATCC 13563)	*Escherichia coli* (ATCC 11775)
X1	1.35 ± 0.0048	1.38 ± 0.0094	1.35 ± 0.0082
X8	1.28 ± 0.0124	1.53 ± 0.0147	1.39 ± 0.0094
X9	1.43 ± 0.0091	1.43 ± 0.0163	1.49 ± 0.0065
X11	1.20 ± 0.0024	1.60 ± 0.0122	1.59 ± 0.0052
X15	1.48 ± 0.0082	1.54 ± 0.0037	1.58 ± 0.0094
L. salivarius LH1F	1.78 ± 0.0124	2.35 ± 0.0163	1.95 ± 0.0082
H	1.85 ± 0.0071	2.10 ± 0.0047	2.10 ± 0.0067

[28] and, consequently, in regulation of the color stability of meat [20,21,29]. Therefore the presence of MetMb reductase proved that H strain can be used in the investigation for color stability of fresh meat.

3.3. Identification of H strain

Serial purification has been conducted to observe the colony and cell morphology of H strain. The colony was a bulge with smooth surface, a diameter of 1–2 mm, showing non-specific pigment after culturing at 37 °C for 48 h. The cells of H strain were Gram-positive, short and long rods shaped after 18–24 h cultivation at 37 °C anaerobically.

3.4. Physiological and biochemical characteristics of H strain

The biochemical reaction characteristics and carbohydrate fermentation test results for identification of H strain were listed in Tables 4 and 5 respectively.

Table 3
H strain enzymes activity.

Enzyme activity	Nitric oxide synthase activity (nmol min^{-1} mL^{-1})	Metmyoglobin reductase activity (nmol min^{-1} mL^{-1})
H strain	34.14 ± 0.153	0.21 ± 0.036

3.5. 16S rRNA sequence identification of H strain

16S rRNA analysis results were shown in Figs. 1 and 2. According to the results of 16S rRNA partial 1464 bp base sequence, H strain was found to be homogenous to *L. salivarius*, and the similarities were more than 99%. And the results of morphological observation, API identification system, its physical, biochemical and molecular biological characteristics were also very close to *L. salivarius*.

From the results above, it can be concluded that H strain belongs to *L. salivarius*, which was stored as CCTCC M2010374 in China Center for Type Culture Collection (CCTCC), Wuhan University, China.

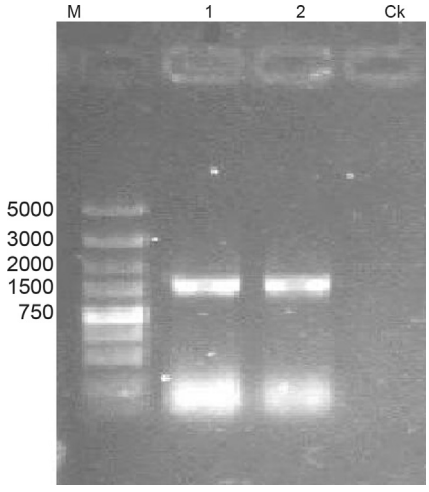

Fig. 1. 16S rRNA PCR for strain H. 1&2, H strain; Ck, negative control; M, DL5000 DNA Marker (bp).

Table 4
Biochemical characteristics of H strain.

	Catalase enzyme	Lactic acid produce	Oxygen demand	Starch hydrolysis	Gelatin liquefaction	V-P test	H_2S produce	Motility
H strain	−	+	facultative	−	−	−	−	−

+: positive reaction; −: negative reaction.

Table 5
Carbohydrate fermentation of H strain.

	Glucose	Gluconate	Lactose	Sucrose	Galactose	Sorbitol	Maltose	Mannitol	Fructose	Trehalose
H strain	+	−	+	+	+	−	+	+	+	+

+: positive reaction; −: negative reaction.

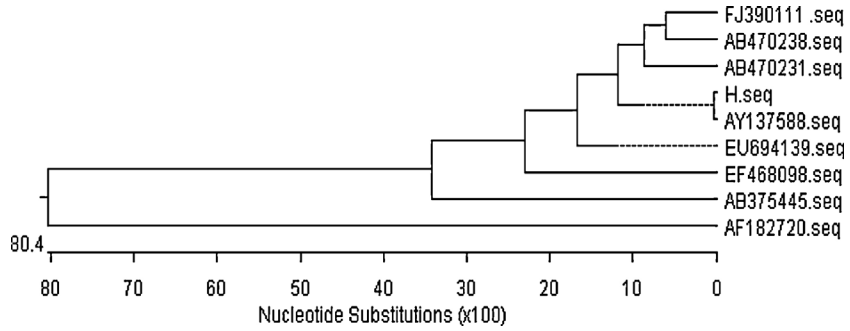

Fig. 2. 16S rRNA sequences alignment for strain H. 1. FJ390111 (*Lactobacillus plantarum*); 2. AB470238 (*Lactobacillus sakei*); 3. AB470231 (*Streptococcus lactis*); 4. H strain; 5. AY137588 (*Lactobacillus salivarius*); 6. EU694139 (*Bulgarian bacillus*); 7. EF468098 (*Lactobacillus acidophilus*); 8. AB375445 (*Lactobacillus curvatus*); 9. AF182720 (*Lactobacillus fermentum*).

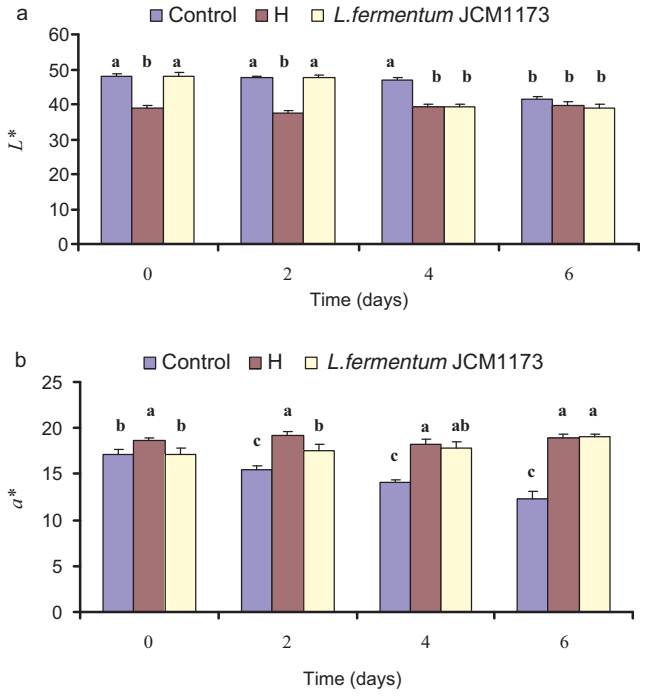

Fig. 3. (a) Meat lightness (L^*) during a 6 days storage period at 4 °C. (b) Meat redness (a^*) during a 6 day storage period at 4 °C. Blue column: control group (0.9% (*m/V*) saline); red column: H strain treated group; yellow column: *L. fermentum* JCM1173 treated group. *a, b, c, different letters at a certain time point mean statistic difference. (b) Meat redness (a^*) during a 6 days storage period at 4 °C.

3.6. Fresh pork color protection ability of LAB at 4 °C for 6 days

3.6.1. Sensory evaluation

With the extension of storage time, the overall acceptability of all groups declined (Table 6). Compared to the control group, H group and *L. fermentum* JCM1173 group maintained the red color after 6 days of storage. H group obtained the highest scores throughout the evaluation period ($P < 0.05$). On day 6, it was decreased by only 35% while the control was above 55% ($P < 0.05$). There was no significant pH changes (data not shown) since all the treated meat samples were stored at 4 °C which resulted in the limted growth or inhibition of microorganisms on the meat surface.

3.6.2. Surface color measurement and metmyoglobin content determination

The CIE L^* and a^* values of treated meat are shown in Fig. 3a and b.

As illustrated in Fig. 3a, the L^* values for *L. fermentum* JCM1173 and the control group declined with the extension of storage time. According to Fig. 3b, a^* values of each treatment group were significantly different. There was a declining trend for the control group while the contrary trend was found for *L. fermentum* JCM1173 group. The a^* values of H group were significantly different ($P < 0.05$) compared to the control group. The L^* and a^* values in H group were not significantly different from the initial readings up to the 6th day of storage, suggesting that overall there was no difference in redness and

Table 6
Sensory evaluation of different treatment groups of meat at 4 °C in 6 days.

	A	B	C	D	A	B	C	D
	Day 0				Day 2			
Control	8.8 ± 0.21^{a}	8.8 ± 0.25^{a}	7.8 ± 0.11^{a}	8.7 ± 0.43^{a}	8.3 ± 0.17^{a}	8.5 ± 0.08^{a}	7.5 ± 0.48^{a}	8.0 ± 0.15^{a}
L. salivarius H	8.9 ± 0.23^{a}	8.9 ± 0.13^{a}	7.8 ± 0.52^{a}	8.6 ± 0.37^{a}	8.4 ± 0.83^{a}	8.7 ± 0.23^{a}	7.9 ± 0.29^{a}	8.2 ± 0.07^{a}
L. fermentum JMC1173	8.9 ± 0.61^{a}	8.9 ± 0.45^{a}	7.9 ± 0.72^{a}	8.7 ± 0.28^{a}	8.5 ± 0.53^{a}	8.3 ± 0.51^{a}	7.9 ± 0.37^{a}	8.1 ± 0.66^{a}
	Day 4				Day 6			
Control	6.7 ± 0.49^{bc}	5.5 ± 0.33^{c}	6.5 ± 0.11^{b}	6.3 ± 0.36^{b}	4.7 ± 0.41^{b}	3.1 ± 0.09^{b}	2.9 ± 0.35^{ab}	3.1 ± 0.27^{c}
L. salivarius H	8.3 ± 0.69^{a}	7.9 ± 0.2^{a}	7.5 ± 0.24^{a}	7.8 ± 0.82^{a}	5.8 ± 0.14^{a}	5.1 ± 0.11^{a}	3.6 ± 0.29^{a}	5.8 ± 0.30^{a}
L. fermentum JMC1173	7.3 ± 0.09^{b}	6.1 ± 0.25^{b}	3.7 ± 0.76^{c}	7.5 ± 0.33^{a}	5.3 ± 0.67^{ab}	4.8 ± 0.82^{a}	3.5 ± 0.47^{a}	4.6 ± 0.51^{b}

A: appearance; B: color; C: smell; D: overall acceptability; Control: 0.9% (w/v) saline. a, b, c, different letters at a certain time point mean statistic difference.

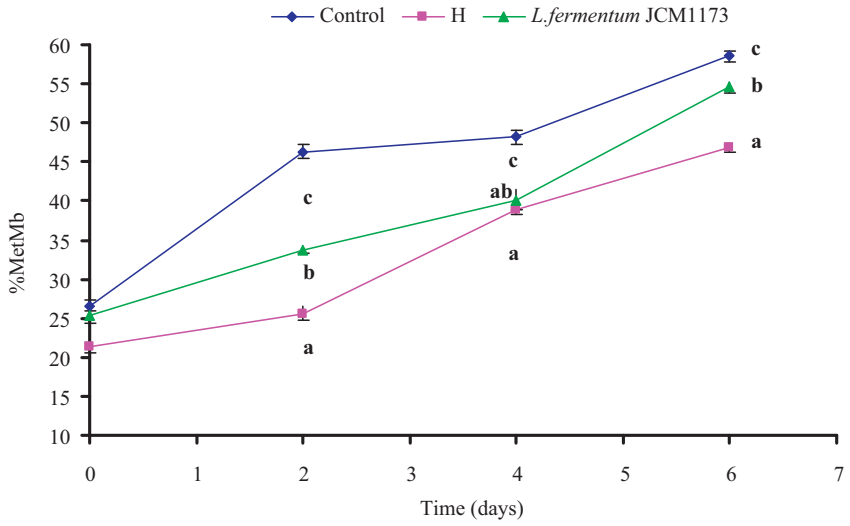

Fig. 4. Metmyoglobin content of fresh pork meat stored for 6 days at 4 °C. Blue line: control group (0.9% (w/v) saline); pink line: H strain treated group; green line: *L. fermentum* JCM1173 treated group. *a, b, c, different letters at a certain time point mean statistic difference.

brightness. This was in agreement with findings of Boles et al. on the color of beef steers upon storage time [30] and the results by Mancini et al. on color stability of beef steaks stored at 1 °C for 9 days [3].

The content of MetMb of each treatment group increased with the extension of storage time at 4 °C (Fig. 4). The control showed the highest MetMb content in all the 6 days during storage at 4 °C, followed by *L. fermentum* JCM1173 treated group. H group showed the least MetMb content of 41%, followed by 50% and 56% for *L. fermentum* JCM1173 and control groups, respectively, on the 6th day.

Taking the results of sensory evaluation, CIE L^* values, and a^* values into consideration, H strains treated group may exert a better effect on meat color stability at 4 °C. This could be a result of the presence of MetMb reductase, reducing MetMb into the redmyoglobin, and NOS, enhancing the formation of NO–Mb. Another reason for the lower MetMb content could be the presence of bacteriocins in *L. salvarius* H strain, which may inhibit the growth of discoloring microorganisms [31].

4. Conclusion

In this study, H strain was isolated, purified and identified from the Chinese dry-cured ham. The strain was identified as *L. salivarius* (CCTCC M2010374) by Gram staining, colony and cell morphology, physiological and biochemical characteristics and by 16S rRNA sequence alignment. According to the results, *L. salivarius* H strain showed the ability to maintain the pork color for 6 days at 4 °C. It is concluded that LAB, such as *L. salivarius* H strain, may provide another option for maintaining fresh meat color apart from MAP and chemical agents. Further studies should be conducted to investigate the mechanism of meat color maintenance by *L. salvarius* H strain.

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